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IDENTIFICATION OF A HIGHLY TRANSFECTABLE CELL LINE PERMISSIVE TO PORCINE EPIDEMIC DIARRHEA VIRUS INFECTION AND REPLICATION

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ABSTRACT

Vero cell line has been routinely used for isolation and propagation of porcine epidemic diarrhea virus (PEDV), but isolation rate of PEDV in Vero cells has been demonstrated low, and the virus may gradually lose host infectivity upon *in vitro* passages. Besides, the entry mechanism of PEDV into Vero cells is also known to be cellular receptor (porcine aminopeptidase N; pAPN; CD13)-independent, which suggests that Vero cells might not be a suitable cell line for studying the interaction of PEDV with its receptor. To explore alternatives, a HEK 293 cell line stably expressing pAPN (HEK 293-pAPN) was established, and its susceptibility to PEDV infection was compared with Vero, HEK 293, and PK-15 cells. Interestingly, cytopathic effects characterized by cell fusion and multinuclear syncytial cells were observed in HEK 293, HEK 293-pAPN, and Vero cells after 48 h of inoculation, but not in PK-15 cells. Moreover, evidence of PEDV replication in these cell lines was also confirmed by immunocytochemistry staining and real-time quantitative PCR analysis. In conclusion, we demonstrated that HEK 293 cells, regardless of overexpression of pAPN molecules, are permissive to PEDV. HEK 293 cell line with its highly transfectable characteristic might not only serve as an alternative tool for studying the entry mechanisms of PEDV, but also a replication permissive cell line for virus rescue such as from the infectious clone of PEDV. Furthermore, the identification of a cell line of human origin, HEK 293, permissive to PEDV raises the concern of possible interspecies transmission.

Keywords: Aminopeptidase N; Permissive cell line; Porcine epidemic diarrhea virus.

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INTRODUCTION

Porcine epidemic diarrhea virus (PEDV), a member of the *Coronaviridae*, is an enveloped, single-stranded positive-sense RNA virus. PEDV first appeared in Europe in early 1970s,[1,2] and has since become an endemic disease throughout Europe and Asia.[3] In late 2013, emerging and re-emerging of new variants of PEDV have been reported in many countries and caused severe diarrhea in piglets, which resulted in a significant economic loss in swine industry.[4,5]

PEDV uses porcine aminopeptidase N (pAPN or CD13) as a cellular receptor for attachment and entry to cells so exhibits restricted cellular tropism.[6,7] APN is a 150-kDa, type II glycoprotein that belongs to a membrane-bound metalloprotease family and can be found in many tissues in both human and animals with different expression levels.[7,8] Up to date, successful *in vitro* viral isolation and propagation of PEDV could only be achieved in African green monkey kidney (Vero) cells and cell lines expressing pAPN.[6,9] However, the mechanism of PEDV entry into Vero cells is pAPN-independent, unlike that into the natural host cells.[10] Moreover, isolation of PEDV in Vero cells has proven difficult; even if PEDV can be isolated from clinical samples, the virus may gradually lose host infectivity upon further passages.[11,12] Therefore, alternative cell lines for isolation of PEDV are needed.

Two cell lines were used for generation of pAPN expressing stable cell lines: human embryonic kidney (HEK 293) cells and pig kidney (PK-15) cells. The HEK 293 cells are popular for their ease of growth and highly transfectable characteristic.[13,14] Expression of the cellular receptor protein, angiotensin-converting enzyme (ACE), has been demonstrated supporting the replication of human coronavirus in HEK293 cells.[15] However, the permissibility of a pAPN-expressing HEK293 to PEDV infection has not been evaluated. Another cell line which is of porcine origin, PK-15, has been reported to be susceptible to PEDV as well as PEDV spike-pseudotyped virus.[16] Hence, we hypothesized that transfection and expression of pAPN on these cell lines might enable these cells to support propagation and replication of PEDV.

In the present study, a HEK 293 cell line stably expressing pAPN (HEK 293-pAPN) has been established. The susceptibility of HEK 293-pAPN cell line to PEDV infection has been compared with those of Vero, HEK 293, and PK-15 cells, respectively, to identify alternative cell lines for viral replication.

MATERIALS AND METHODS

Virus

The parental PEDV strain, TW-Pintung-52 (Genbank accession No. KP276252), was isolated from intestinal homogenates from a seven day-old suckling pig in early 2014 in Taiwan and adapted to Vero cell as previously described.[17] Viral infection and propagation were confirmed by daily observation of cytopathic effects (CPEs), real time reverse transcription quantitative PCR (RT-qPCR), and indirect immunocytochemistry staining (ICC) using the in-house mouse anti-PEDV N monoclonal antibody (mAb). Briefly, Vero cells (Vero, C1008) were cultured overnight (O/N) to 80% confluence and washed twice with the modified post-inoculation medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), and 10 μg/mL of trypsin. After two blind passages, the infected cells started showing CPEs characterized by cell fusion, syncytial cells formation, and cell detachment. To prepare the viral stock for challenge, PEDV-infected cells were disrupted by freeze-thawing once to harvest the virus at 48 hours post-infection (HPI) when more than 90% of cells showing CPEs.[12,17] The titer of the viral stock was 5×10^5 $TCID_{50}$/mL.

CONSTRUCTION AND EXPRESSION OF PORCINE AMINOPEPTIDASE N (pAPN)

Two cell lines, HEK 293 and PK-15, were used for generation of stable cell lines expressing pAPN. In brief, total RNA was extracted from enterocytes of the small intestine of a seven day-old suckling piglet using the QIAamp RNeasy Mini Kit (Qiagen, Hilder, Germany), and the pAPN sequence was amplified by RT-PCR using a forward primer APN3.1F 5′-CAC CAT GGC CAA GGG ATT CTA CAT TTC-3′; and a reverse primer APN3.1R 5′-AAA GCT GTG CTC TAT GAA CCA ATT CAA C-3′. The PCR product was subcloned into the pcDNA 3.1-V5-His vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Plasmid DNA was purified by a plasmid DNA purification kit (Qiagen, Hilden, Germany) for subsequent transfection. HEK 293 and PK-15 cells were both transfected with pAPN plasmids (designated as HEK 293-pAPN and PK-15-pAPN) for 48 h using the

PolyJet (SignaGen, Gaithersburg, MD, USA) transfection reagent. The transfection medium was replaced with fresh growth medium at 48 h post-transfection. The transfected cell lines were selected using DMEM supplemented with 10% FBS containing 750 μg/mL G418 (Invitrogen) for two weeks to establish pAPN stably expressing cell line. Expression of pAPN in the transfected cells was confirmed by ICC after two weeks of transfection. To perform the pAPN blocking assay, the pAPN protein was further purified by metal-binding affinity of histidine (GE healthcare, Little Chalfont, UK), and desalted and concentrated by VivaspinTM (GE healthcare). The level of expression and the size of the expressed pAPN protein were confirmed by western blot and ICC using anti-V5 (Invitrogen).

PEDV INFECTION IN HEK 293, HEK 293-pAPN, PK-15 AND PK-15-pAPN CELL LINES

The Vero, HEK 293, HEK 293-pAPN, PK-15, and PK-15-pAPN cell lines were maintained in DMEM supplemented with 10% FBS in 12-well tissue culture plates at 37°C for 24 h. These cells were inoculated either with the PEDV (TW-Pintung-52 strain) stock virus or 1:10 dilution of intestinal homogenates from PEDV-infected piglets at 37°C with 5% CO_2 for 2 h. The supernatant was then removed, washed twice with PBS, and replaced by modified post-inoculation medium containing DMEM supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), and different concentrations of trypsin (0.25, 0.5, 1 or 10 μg/mL) for five days. At 0, 24, 48, 72, 96, and 120 HPI, CPEs were evaluated and the cells were fixed in 80% acetone at −20°C for 10 min for ICC. For viral RNA detection, 140 μL supernatants were collected and stored at −20°C for later PEDV RNA extraction and SYBR Green-based real-time qPCR determination.

IMMUNOCYTOCHEMISTRY STAINING

To detect the expression of pAPN, cells were fixed in 80% acetone at −20°C for 10 min, and stained with anti-V5 antibody (Invitrogen) or anti-CD13 antibody (Abcam, Milton, Cambridge, UK) for 1 h at room temperature (RT). After three times of PBS washing, the acetone-fixed cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody for another 1 h at RT. For viral antigen detection, infected cells were fixed using the previous described method, and stained with anti-PEDV N protein monoclonal antibody for 1 h at RT. After three times of PBS washing, cells were incubated with HRP-conjugated goat anti-mouse antibody for 1 h at RT. After incubation and three times of PBS washing, these labeled cells were then exposed to DAB/chromogen using a peroxidase DAB substrate kit (Dako, Carpinteria, California, USA) according to the manufacturer's instructions.

RNA EXTRACTION AND SYBR GREEN-BASED REAL-TIME RT-qPCR

Viral RNA was extracted from 140 μL of cultural supernatants using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's protocols. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions with the following thermal cycling conditions: 25°C (10 min), 42°C (15 min), and 85°C (5 min). The following primers were used for PEDV detection according to previously published primers[13]: PEDN219F/5′-GCA TTT CTA CTA CCT CGG AAC A-3′ and PEDN557R/5′-CTC CAC GAC CCT GGT TAT TT-3′. The real time qPCR was performed using SYBR® Advantage® qPCR Premix (Clontech, California, USA) under following conditions: 95°C for 5 s, 60°C for 30 s, 95°C for 15 s and totally repeated 45 cycles using the Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). All real-time RT-qPCR reactions were performed in triplicate. Fluorescent detection was at the end of each cycle. Melting curve analysis was used for identification of specific PCR products. After the last cycle, the temperature was increased to 94°C, then decreased to 75°C and slowly increased to 94°C at a rate of 0.1°C per second, with continuous fluorescence monitoring. Positive and negative controls (double-distilled water) were included in each set. Serial 10-fold dilutions of known amount of plasmid pCR-XL-TOPO DNA (Thermo Fisher Scientific, Waltham, MA, USA) containing PEDV N gene were used as positive control and to generate the standard curve. The genome equivalent (GE) copies were calculated by converting molecular weight unit of double-stranded plasmid into numbers of DNA molecules. Standard curves were produced as averaged cycle threshold (Ct) versus GE copies/mL over an 8-log range from 0 to 10^8. The efficiency of the real-time RT-qPCR was 92.2% based on the slope of the standard curve. The average of the correlation coefficient of the standard

curves was 0.9. The detection limit of the real-time RT-qPCR was about 60 GE copies/mL using the plasmid encoding PEDV N gene as a standard (data not shown). The GE copies of three replicates of viral RNA from culture supernatant were calculated according to the standard curve.

STATISTICAL ANALYSIS

Statistical analysis was performed using Duncan's multiple range test in statistical analysis system (version 9; SAS Institute Inc., Cary, NC, USA). The average GE copies among three cell lines at each time point were compared.

RESULTS

Establishment of pAPN Expressing Stable Cell Lines

Expression of pAPN in HEK 293-pAPN cells was confirmed by ICC using the monoclonal antibody against hAPN (Fig. 1(C)) or the V5 epitope at the C-terminal of the pAPN protein two weeks post-transfection (Fig. 1(E)). The percentage of HEK 293-pAPN cells expressing the V5 epitope was about 75–80% (Fig. 1 (E)). Interestingly, weak positive signals were also recognized in HEK 293 cells by the hAPN antibody (Fig. 1 (B)), but not by the anti-V5 antibody (Fig. 1(D)). As for PK-15-pAPN and PK-15 cells, less than 1% cells were stained weak positive by using either anti-APN antibody or anti-histidine antibodies.

PERMISSIVENESS OF VERO, PK-15, HEK 293, AND HEK 293-pAPN CELL LINES TO PEDV

A significant trypsin-induced cytotoxicity was observed in both HEK 293 and HEK 293-pAPN cells treated with post-inoculation medium containing the original concentration of 10.0 μg/mL trypsin. Therefore, the concentration of trypsin causing minimal cytotoxicity was examined. The pilot study indicated that these cells maintained in the post-inoculation medium containing 0.25 μg/mL trypsin showed minimal cytotoxicity. CPEs characterized by cell fusion and formation of

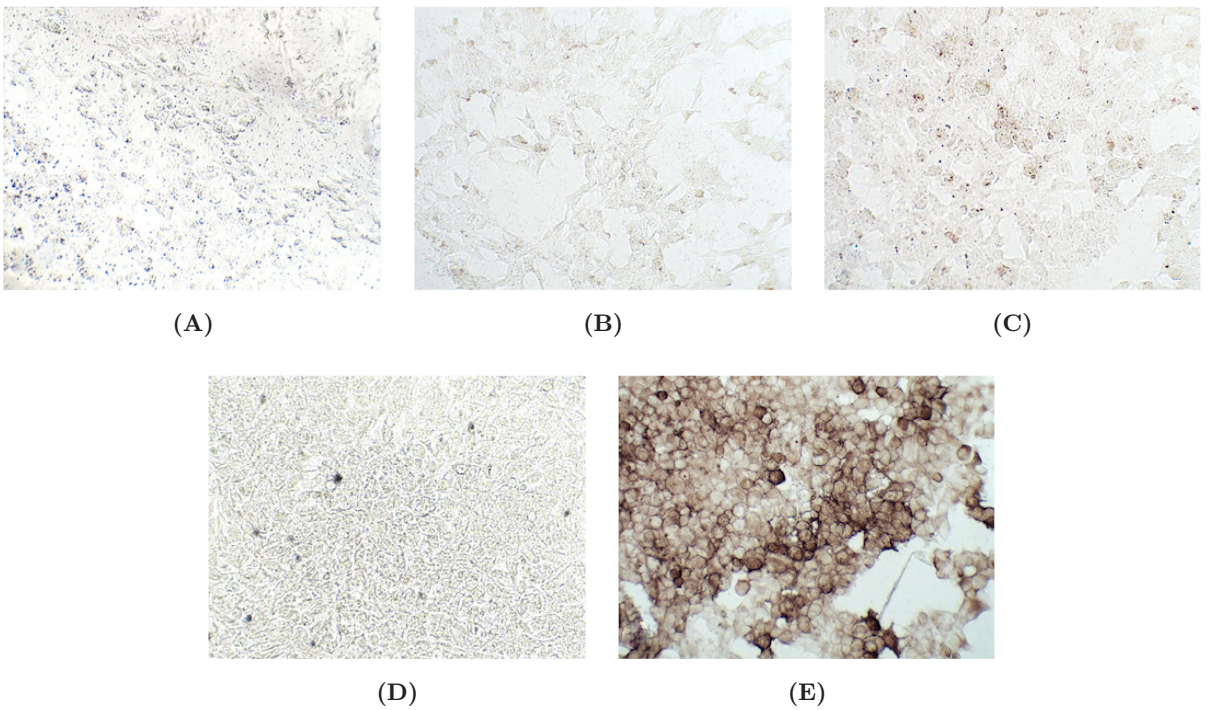

Fig. 1 (Color online) Detection of pAPN protein expression in HEK 293 and HEK 293-pAPN cells by ICC using anti-CD13 or anti-V5 antibody, followed by DAB staining (A) Negative control of HEK 293 cells stained with HRP-conjugated goat anti-mouse antibody (B) HEK 293 cells stained with anti-CD13 antibody (C) HEK 293-pAPN cells stained with anti-CD13 antibody (D) Negative control of HEK 293 cells stained with anti-V5 antibody (E) HEK 293-pAPN cells stained with anti-V5 antibody. (Magnification × 200).

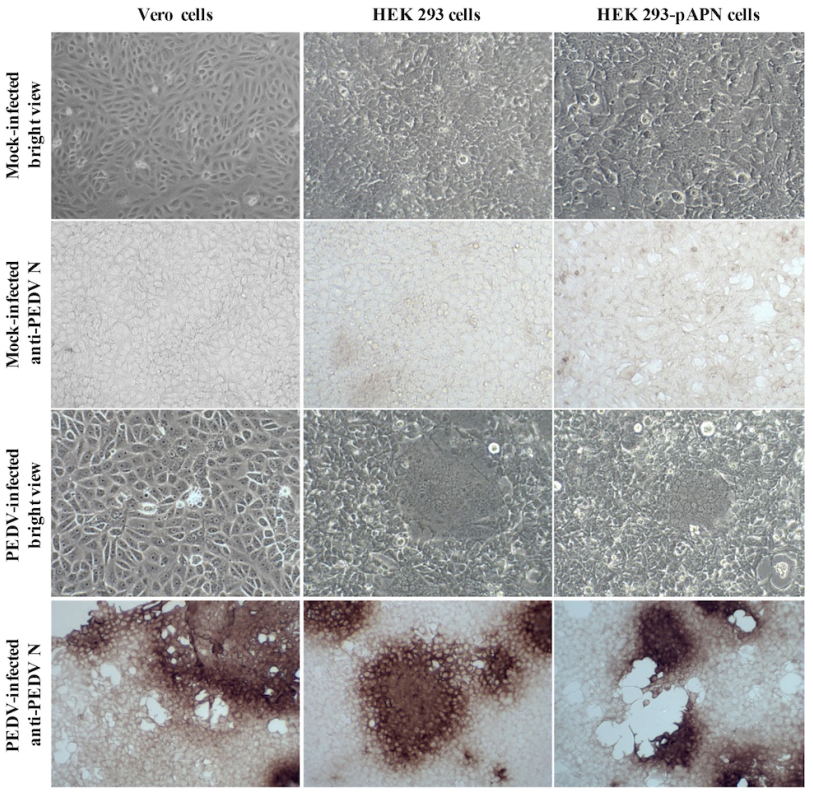

Fig. 2 (Color online) CPEs and antigen detection of mock-infected and PEDV-infected Vero, HEK 293, and HEK 293-pAPN cells by light microscopic examination or ICC using anti-PEDV N antibody, followed by DAB staining. Distinct CPEs with cell fusion and syncytial cells were observed at 96 HPI. (Magnification × 200).

multinuclear syncytial cells were observed in HEK 293 and HEK 293-pAPN cells maintained in post-inoculation medium containing 0.25 μg/mL trypsin at 48 HPI (Fig. 2). However, by using this post-inoculation medium, typical CPEs were difficult to be detected in Vero cells under the light microscopic examination, but positive intracytoplasmic signals could be detected by ICC (Fig. 2). Although fewer CPEs were observed in Vero cells, post-inoculation medium containing 0.25 μg/mL trypsin was used in the present study to access the permissiveness of HEK 293 or HEK 293-pAPN cells to PEDV replication.

To evaluate the replication ability of PEDV in Vero, HEK 293, and HEK 293-pAPN cell lines, viral RNA of PEDV in supernatants of different cell lines was quantified by SYBR Green-based real-time RT-qPCR at different time points (Fig. 3). At 24 HPI, except for HEK-293, increased viral loads in about 1–2 $\log_{10}$ GE copies/mL were detected in the supernatant of PEDV-infected Vero and HEK 293-pAPN cell lines. None of these PEDV-inoculated cell lines showed detectable CPEs at 24 HPI. At 96 HPI, significant CPEs characterized by formation of syncytial cells in all PEDV-inoculated cell lines were observed. At 120 HPI, elevated viral loads of 4.9 $\log_{10}$ GE copies/mL in Vero, 4.8 $\log_{10}$ GE copies/mL in HEK 293 cells, and 4.5 $\log_{10}$ GE copies/mL in HEK 293-pAPN cells were detected. No significantly statistical differences in average GE copies among three cell lines at each time point were noted.

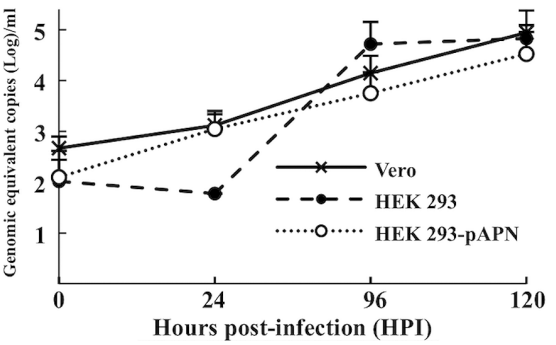

Fig. 3 (Color online) PEDV GE copies/mL in supernatants of PEDV-infected Vero, HEK 293, and HEK 293-pAPN cell lines detected by real time RT-qPCR. All reactions were performed in triplicate. The detection limit of the real time RT-qPCR was 60 GE copies/mL in this study. Error bars represent standard deviation.

To evaluate the role of APN molecules contributing to the entry of PEDV into these cell lines, the pAPN proteins were further purified from the HEK 293-pAPN cell line and used to compete and block the receptor binding protein on the surface of PEDV. Our result indicated that pre-incubation of PEDV with pAPN did not inhibit viral replication in Vero, HEK 293, and HEK 293-pAPN cells. The role of APN in the entry of PEDV into Vero, HEK 293, or HEK 293-pAPN cells were inconclusive. Furthermore, several attempts were made to isolate PEDV from clinical specimens using these HEK 293 cell lines. No typical PEDV-associated CPE and ICC positive signals, but massive cytotoxicity, were detected in HEK 293 or HEK 293-pAPN cells inoculated with intestinal homogenates.

DISCUSSION

In order to improve the laboratory isolation rate and explore alternative cells for studying the viral entry mechanisms of PEDV, a HEK 293 cell line stably expressing pAPN has been successfully established. We were surprised that regardless of pAPN expression status, both HEK 293 and HEK 293-pAPN cells are permissive to PEDV infection and replication. Evidence of PEDV replication in these cell lines was also confirmed by ICC and real-time RT-qPCR. Our findings of permissiveness of the cell lines for PEDV entry and replication suggested that these cells might serve as alternative tools for studying PEDV pathogenesis, entry mechanisms, and virus rescue such as from the infectious clone of PEDV.

It has been demonstrated that PEDV can use both pAPN and human APN (hAPN) as its receptor.[16] PEDV is able to infect cell lines expressing endogenous pAPN,[16] and cell lines expressing exogenous pAPN such as Madin–Darby canine kidney (MDCK) cells,[7] swine testis cells,[6] or duck intestinal epithelial cells.[9] PEDV can also infect cells expressing endogenous hAPN such as human liver (Huh-7) cells, and MDCK cells expressing exogenous hAPN.[16] Hence, we speculated that the permissiveness of HEK 293 cells to PEDV might be due to the expression of endogenous hAPN detected by ICC in the present study. To prove the role of the endogenous hAPN in PEDV replication in HEK 293 cells, we then purified the pAPN protein and pre-incubated it with PEDV to compete the receptor binding domain on the surface of PEDV. However, replication of PEDV in HEK 293 cells was not able to be blocked by this approach. Other than APN molecules, we speculated that the recently identified sugar co-receptor of PEDV might play a role in entry of PEDV into HEK 293 cells as well.[16,18]

Although, trypsin has been routinely used to enhance PEDV infectivity in Vero cells,[19] trypsin-induced cytotoxicity in HEK 293 cells was a concern in our study. We found that the routinely used concentration of 10 μg/mL trypsin caused no cytotoxicity in Vero and PK-15 cells, but induced significant cytotoxicity in both HEK 293 and HEK 293-pAPN cells. After adjusting the concentration of trypsin in the post-inoculation medium, we determined that 0.25 μg/mL of trypsin showed the slightest cytotoxicity to both cell lines under mock-infected condition while inducing prominent PEDV-associated CPEs after PEDV inoculation. However, severe and non-specific cytotoxicity was found when attempting to isolate PEDV from clinical samples using HEK 293 cell lines in post-inoculation medium with 0.25 μg/mL trypsin. Cytotoxicity was observed in cells inoculated with several clinical samples, which might be due to the enteric digestive enzymes in the intestinal contents. The isolation rate of PEDV was only 4.0–10.2% in previous studies.[11,20] A better isolation method and alternative enzyme resistant cell lines are still needed for improving the viral isolation rate of PEDV.

In our study, no CPEs and viral positive signals were observed in both PK-15 and PK-15-pAPN cells after inoculation of PEDV, which was not consistent with a previous study.[16] It is suspected that the density of APN receptor played an important role in viral permissibility.[6] Non-permissiveness of PK-15 cell line to PEDV could be attributed to weak signals of the APN positive cells and low transfection efficiency in this cell line. Less expression of pAPN proteins in PK-15-pAPN cell line as compared with HEK 293 cell line might also result in its non-permissiveness to PEDV.

The reverse genetics system is popular for manipulation of viral genome and generation of mutants for genetic analysis and pathogenesis study. Here, we presented that HEK 293 cell line is highly transfectable and can be used for virus packaging and PEDV recovery. It might serve as a good cell line for reverse genetic studies. Identification of human cells permissive to PEDV, HEK 293 cells in the present study or Huh-7 cells as previously described, highlights the possibility of interspecies transmission of this virus.[16,21]

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